

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 31/00	A2	(11) International Publication Number: WO 00/23060 (43) International Publication Date: 27 April 2000 (27.04.00)
(21) International Application Number: PCT/US99/24371 (22) International Filing Date: 19 October 1999 (19.10.99) (30) Priority Data: 60/104,875 20 October 1998 (20.10.98) US (71) Applicant: BOEHRINGER INGELHEIM PHARMACEUTICALS, INC. [US/US]; 900 Ridgebury Road, Ridgefield, CT 06877 (US). (72) Inventors: ERICKSON, David; Apartment 13, 136 Deer Hill Avenue, Danbury, CT 06810 (US). GROB, Peter, M.; 2 Meadowbrook Road, New Fairfield, CT 06812 (US). HOFFMAN, Ann, F.; RR1 Box 319J, Cascade Mountain Road, Amenia, NY 12501 (US). WARREN, Thomas, C.; 426 Bennetts Farm Road, Ridgefield, CT 06877 (US). (74) Agents: RAYMOND, Robert et al.; Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, Ridgefield, CT 06877 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: METHOD OF TREATING IMMUNOLOGICAL DISORDERS MEDIATED BY T-LYMPHOCYTES		
(57) Abstract The use of soluble epoxide hydrolase inhibitors in the treatment of T-lymphocyte mediated immunological disorders is described either as monotherapies or as part of combination therapies for such disorders.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

METHOD OF TREATING IMMUNOLOGICAL DISORDERS
MEDIATED BY T-LYMPHOCYTES

Cross-Reference to Related Applications

This application is a continuation in-part of Provisional Patent Application 60/104,875, filed October 20, 1998.

Technical Field of the Invention

The present invention relates to methods of treating T-lymphocyte mediated immunological disorders by administration of a therapeutic amount of an inhibitor of soluble epoxide hydrolase. Because of selective immunomodulating properties, such inhibitors and their pharmaceutical compositions are particularly well suited for preventing and treating autoimmune diseases.

Background of the Invention

It has been well established that T-lymphocytes play an important role in regulating immune response (Powrie and Coffman, Immunology Today, 14:270-274;1993). Indeed, activation of T-lymphocytes is often the initiating event in immunological disorders. Following T-lymphocyte stimulation, there is an influx of calcium that is required for T-lymphocyte activation. Upon activation, T-lymphocytes produce cytokines, such as interleukin-2 (IL-2). The release of IL-2 is critically important since it is required for T-lymphocyte proliferation, differentiation, and effector function. Clinical studies have shown that interference with IL-2 activity effectively suppresses immune response *in vivo* (Waldmann, Immunology Today, 14:264-270;1993). Accordingly, agents which inhibit T-lymphocyte activation and subsequent IL-2 production, or block the activity of IL-2 are therapeutically useful for selectively suppressing immune response in a patient in need of such immunosuppression. Furthermore, it has been established that elevated intracellular calcium levels brought about by an influx of extracellular calcium is necessary to initiate T-cell activation and IL-2 gene transcription (Jy et al., Biochimica et Biophysica Acta,

983:153-160;1989, Chung et al., Br. J. Pharmacology, 113:861-868;1994, Timmerman et al., Nature, 383:837-840;1996). Therefore, direct or indirect inhibition of Ca^{+2} influx would inhibit IL-2 production.

It is now known that distinct subsets of CD4^{+} helper cells are responsible for driving the two types of immune effector function; cell-mediated and humoral immune responses (Abbas et al., Nature, 383:787-793;1996). Cell-mediated responses, including activation of macrophages and induction of cytotoxic T-lymphocytes, are driven by $\text{T}_{\text{H}}1$ CD4^{+} helper cells where cytokines such as IL-2, interferon-gamma ($\text{IFN}\gamma$) and tumor necrosis factor-beta ($\text{TNF}\beta$) are released. The humoral response, characterized by antibody production, is driven by $\text{T}_{\text{H}}2$ CD4^{+} helper cells where the cytokines interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-10 (IL-10) are produced.

In concert with IL-2 expression, the production of these cytokines and *in vivo* emergence of $\text{T}_{\text{H}}1$ and $\text{T}_{\text{H}}2$ helper cells correlates with immunological disorders. Like IL-2, the induction of these cytokines by T-lymphocytes has been shown to be calcium-dependent ($\text{IFN}\gamma$: Kaldy, et al., European J. Immunology, 25:889-895;1995; $\text{TNF}\beta$: Sung et al., J. Experimental Medicine, 167:937-953;1988; IL-4: Rooney, et al., EMBO J., 13:625-633;1994; IL-5: Blumenthal, et al., J. Biological Chemistry, 274:12910-12916;1999; IL-10: Minai, et al., Cellular Immunology, 172:200-204;1996). Agents which prevent calcium influx into activated T-lymphocytes are expected to inhibit production of these cytokines and thereby diminish the immune response. Such agents would be expected to exhibit therapeutic utility with regards to treating immunological disorders.

Previous attempts to interfere with the activity of IL-2 were made by using cytokine antagonists, monoclonal antibodies, toxins and other biologics which seek to prevent IL-2 from binding to its receptor (Waldmann, Immunology Today, 14:264-270;1993). More recent attempts to inhibit IL-2 production at the T-lymphocyte level were made by blocking the expression of IL-2 mRNA with glucocorticoids, cyclosporin A or FK506. However, to date, the reported compounds suffer from several disadvantages such as low potency, poor *in vivo* activity, toxicity and poor oral bioavailability. Cyclosporin A and

FK506 have proven to be very effective therapeutic agents for treating immunological disorders and exert their activity by inhibition of the calcium-dependent phosphatase, calcineurin (Rao, Immunology Today, 15:274-281;1994). Calcineurin plays an essential role in regulating T-lymphocyte cytokine expression, including IL-2, and is the primary target of cyclosporin A and FK506. Since calcineurin activity is calcium-dependent, agents which inhibit calcium influx are expected to inhibit the enzymatic activity of this phosphatase. Inhibition of calcineurin will prevent cytokine production by T-lymphocytes and thereby achieve the beneficial therapeutic utility exhibited by cyclosporin A and FK506 in treating immunological disorders.

Epoxide hydrolases are a group of enzymes ubiquitous in nature, detected in species ranging from plants to mammals. These enzymes are functionally related in that they all catalyze the addition of water to an epoxide, resulting in a vicinal diol. Epoxide hydrolases are important metabolizing enzymes in living systems. Epoxides are reactive species and once formed are capable of undergoing nucleophilic addition. Epoxides are frequently found as intermediates in the metabolic pathway of xenobiotics. Thus in the process of metabolism of xenobiotics, reactive species are formed which are capable of undergoing addition to biological nucleophiles. Epoxide hydrolases are therefore important enzymes for the detoxification of epoxides by conversion to their corresponding, non-reactive 1,2-diols.

In mammals, several types of epoxide hydrolases have been characterized including soluble epoxide hydrolase (sEH), also referred to as cytosolic epoxide hydrolase. The epoxide hydrolases differ in their specificity towards epoxide substrates. For example, sEH is selective for aliphatic epoxides such as epoxide fatty acids while microsomal epoxide hydrolase (mEH) is more selective for cyclic and arene oxides. Epoxide hydrolases have been found in all tissues examined in vertebrates, including heart, kidney and liver (Vogel-Bindel et al., Eur J. Biochemistry, 126:425-431;1982; Schladt et al., Biochem. Pharmacology, 35:3309-3316;1986). Epoxide hydrolases have also been detected in human blood components including lymphocytes (e.g. T-lymphocytes),

monocytes, erythrocytes, platelets and plasma. In the blood, most of the sEH detected was present in lymphocytes (Seidegard et al., Cancer Research, 44:3654-3660;1984).

Some diols produced by sEH have potent biological effects. Soluble epoxide hydrolase metabolism of epoxides produced from linoleic acid (leukotoxins) produces leukotoxin diols. These diols were shown to be toxic to cultured rat alveolar epithelial cells, increasing intracellular calcium levels, increasing intercellular junction permeability and promoting loss of epithelial integrity (Moghaddam et al., Nature Medicine, 3:562-566;1997). Therefore these diols could contribute to the etiology of diseases such as adult respiratory distress syndrome. Hammock et al. have disclosed the treatment of inflammatory diseases, in particular adult respiratory distress syndrome and other acute inflammatory conditions mediated by lipid metabolites, by the administration of inhibitors of epoxide hydrolase (WO 98/06261, 1998). Hammock et al., have also disclosed that phenyl chalcone oxides are epoxide hydrolase inhibitors.

Summary of the Invention

The present invention provides a method of treating immunological disorders, by administration of a therapeutic amount of an inhibitor of soluble epoxide hydrolase, either alone or in combination with other therapeutic agents.

Brief Description of the Drawing

Fig. 1 shows the percentage of inhibition of IL-2 Production and sEH for select compounds.

Detailed Description of the Invention

As used herein, each of the following terms, used alone or in conjunction with other terms, are defined as follows (except where noted to the contrary):

The term "immunological disorder" includes, but is not limited to (1) autoimmune diseases, (2) disorders associated with T-lymphocyte mediated immune responses, (3) transplantation; allograft or xenograft rejection and (4) graft versus host disease.

The term "autoimmune disease" includes, but is not limited to the following diseases: Hashimoto's thyroiditis, systemic lupus erythematosus, Goodpasture's syndrome, pemphigus, receptor autoimmunity (including but not restricted to Grave's disease, myasthenia gravis, insulin resistance), autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, autoimmune hepatitis, rheumatoid arthritis, scleroderma, mixed connective tissue disease, polymyositis, pernicious anemia, idiopathic Addison's disease, glomerulonephritis, bullous pemphigoid, Sjogren's syndrome, diabetes mellitus, adrenergic drug resistance, chronic active hepatitis, primary biliary cirrhosis, vitiligo, vasculitis, post-MI, cardiomyopathy syndrome, urticaria, atopic dermatitis and asthma.

The term "disorders associated with T-lymphocyte mediated immune responses" includes, but is not limited to the following diseases: multiple sclerosis, uveitis, allergic encephalomyelitis, glomerulonephritis, psoriasis, erythemas, cutaneous eosinophilias, acne, keratoconjunctivitis, vernal conjunctivitis, keratitis, dystopia sarcoidosis, obstructive airways disease, bronchitis, gastric ulcers, vascular damage caused by bowel disease and thrombosis, ischemic bowel diseases, necrotizing enterocolitis, intestinal lesions due to thermal burns or leukotriene B4-mediated diseases, Crohns disease, ulcerative colitis, rhinitis, eczema, interstitial nephritis, diabetic nephropathy, osteoporosis, sarcoidosis, fibroid lung, photoallergic sensitivity, arteriosclerosis, atherosclerosis, polyarteritis, scleroderma, endotoxin-shock, chronic and ischemic acute renal insufficiency and Guillain-Barré syndrome.

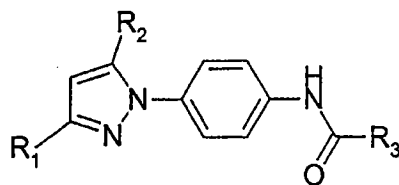
The term "patient" refers to a warm-blooded animal, and preferably a human.

The term "treatment" refers to either the alleviation of the physical symptoms of a disease or an improvement in the physiological markers used to measure progression of a disease state.

The term "pharmaceutically acceptable carrier" or "pharmaceutically acceptable adjuvant" refers to a non-toxic carrier or adjuvant that may be administered to a patient together with a compound of this invention and which does not destroy the pharmacological activity of that compound.

The term "therapeutically effective amount" refers to an amount effective in preventing or reducing the likelihood of initial onset or progression of an immunological disorder in a patient susceptible to such disorder, and in suppressing the immune response of a patient in need of such treatment. For example, suppressed immunity can be readily measured by observing the degree of inhibition of IL-2 production in human T-lymphocytes (peripheral blood lymphocytes) by known techniques.

The discovery, described herein, of a novel mechanism for inhibiting a T-lymphocyte mediated immune response was achieved by identifying the molecular target of a novel series of compounds which block the influx of calcium into stimulated Jurkat cells and block the production of IL-2 by human T-lymphocytes (Betageri et al., U.S. S. N. 09/324,933, filed June 3, 1999, the subject matter and disclosures of which are incorporated herein by reference). A similar group of compounds is disclosed in WO 99/19303A1 (H. Kubota et al., April 22, 1999). It is expected that such compounds would also inhibit sEH. A photoaffinity probe (4-azido-*N*-[4-(3,5-bis-trifluoromethyl-pyrazol-1yl)-phenyl]-benzamide) was designed, prepared and specific photoaffinity labeling was achieved in both whole cells and cell lysates (Jurkat cells, an immortalized human cell line). A putative molecular target in Jurkat lysates was chromatographically enriched, resolved by SDS-PAGE and identified as sEH (Sandberg and Meijer, *Biochem. Biophys. Res. Commun.* 221:333-339; 1996). Compounds selected from the chemical series disclosed in Betageri, et al., which exhibit a range of inhibition against calcium influx and IL-2 production (Table 1), were then tested and the active compounds were also found to be potent and direct inhibitors of sEH (Tables 2-3 and Figure 1).

Table 1: Structures of Compounds in Tables 2 and 3, and Figure 1

Compound Number	R ₁	R ₂	R ₃
1	3-pyridinyl	Et	3-pyridinyl
2	MeOCH ₂	CF ₃	3-pyridinyl
3	i-Pr	Et	3-pyridinyl
4	Et	Et	3-pyridinyl
5	Et	i-Pr	3-pyridinyl
6	CF ₃	2-oxazolidinyl	3-pyridinyl
7	Me	Me	3,5-dimethyloxazol-4-yl
8	MeOCH ₂	Me	3-pyridinyl
9	3-pyridinyl	CF ₃	2-chloropyridin-4-yl
10	3-pyridinyl	CF ₃	3-pyridinyl
11	CF ₃	CF ₃	3-pyridinyl

Table 2: Inhibitory Profile of Compound 1

Assay	Activity of Compound 1 (IC₅₀, percent inhibition or dose yielding significant inhibition <i>in vivo</i>)
Inhibition of soluble Epoxide Hydrolase s-NEPC colorimetric assay	60 nM
Inhibition of cytosolic soluble Epoxide Hydrolase EET HPLC assay (Method I)	51% inhibition at 250nM; Jurkat cell cytosol 49% inhibition at 250nM; human liver cytosol
Inhibition of calcium influx into Jurkat cells	300 nM
Inhibition of IL-2 reporter assay	80 nM
Inhibition of IL-2 release by human PBMCs	20 nM
Inhibition of IL-4 release by human PBMCs	16 nM
Inhibition of IFN γ release by human PBMCs	22 nM
Inhibition of SEB induced IL-2 in mice; <i>in vivo</i>	100 mg/kg p.o.; p<0.001
Inhibition of Allogeneic Cell Transplants in mice, <i>in vivo</i>	100 mg/kg p.o.; p<0.005

Table 3: Correlation between Inhibition of sEH, Ca⁺⁺ Influx, and IL-2 Reporter Gene Expression

Compound Number	Compound IC ₅₀ (nM)		
	SEH Inhibition (s-NEPC assay)	Jurkat Ca ⁺⁺ influx	IL-2 Reporter Assay
2	36	471	422
3	464	214	190
4	591	434	600
5	748	796	2000
6	1053	1918	2710
7	5% @ 10 uM	10,000	18,000
8	66% at 10 uM	11,725	19,080
4-Phenyl Chalcone Oxide	55	3000 [†]	ND

ND: Not determined

[†]Note: 4-phenyl chalcone oxide exhibits a significant rate of spontaneous decomposition in aqueous reducing environments and therefore fails to achieve high potency in cellular assays which require incubation times of > 5 minutes. For this reason, the pre-incubation time was restricted to 2 minutes. (Morisseau et. al., *Arch. Biochem. Biophys.*, 356:214;1998 and Mullin and Hammock, *Arch. Biochem. Biophys.*, 216:423;1982.

We further discovered that 4-phenyl chalcone oxide, a member of an unrelated class of soluble epoxide hydrolase inhibitors, the chalcone oxides, exhibits immunosuppressive activity as evidenced by inhibition of calcium influx in the Jurkat cell calcium assay (Table 3).

An inhibitor of sEH blocks T-lymphocyte activation and subsequent cytokine production by inhibiting extracellular calcium influx. Inhibiting cytokine production is therapeutically useful for selectively suppressing immune function. The result of such suppressed immunity includes reduced immunoglobulin synthesis, cell proliferation of peripheral blood lymphocytes and cellular immune response without serious toxicity or undesired side effects. Thus, the inhibition of cytokine production brought about by inhibition of sEH is an attractive means for preventing and treating a variety of immunological disorders. Other disorders associated with cytokine mediated immune response may also be treated with an inhibitor of sEH.

An inhibitor of sEH may be administered in any conventional dosage form in any conventional manner. Such methods of treatment, including their dosage levels and other requirements, may be selected by those of ordinary skill in the art from available methods and techniques. For example, a compound of this invention may be combined with a pharmaceutically acceptable carrier or adjuvant for administration to a patient in need of such treatment in a pharmaceutically acceptable manner and in an amount effective to treat (including lessening the severity of symptoms) the immunological disorder.

An inhibitor of sEH may be administered alone or in combination with other therapeutics. Advantageously, such combination therapies utilize lower dosages of therapeutics, thus avoiding possible toxicity and adverse side effects incurred when those agents are used as monotherapies.

According to this invention, the inhibitor of sEH and the pharmaceutical compositions thereof may be administered to a patient in any conventional manner and in any pharmaceutically acceptable dosage form, including, but not limited to, intravenously, intramuscularly, subcutaneously, intrasynovially, sublingually, transdermally, orally, topically or by infusion or inhalation.

Dosage forms include pharmaceutically acceptable carriers and adjuvants known to those of ordinary skill in the art. These carriers and adjuvants include, for example, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, buffer substances, water, salts or electrolytes and cellulose-based substances. Dosage forms include tablet, capsule, caplet, liquid, solution, suspension, emulsion, lozenges, syrup, reconstitutable powder, granule, suppository and transdermal patch. Methods for preparing such dosage forms are known (see, for example, Ansel and Popovich, Pharmaceutical Dosage Forms and Drug Delivery Systems, 5th ed., Lea and Febiger, 1990). Dosage levels and requirements are well recognized in the art and may be selected by those of ordinary skill in the art from available methods and techniques suitable for a particular patient. Lower or higher doses may be required depending on particular factors. For instance, specific dosage and treatment regimens will depend on factors such as the patient's general health

profile, the severity and course of the patient's disorder or disposition thereto and the judgment of the treating physician.

Compound 1 is a representative member from a specific chemical series of sEH inhibitors (Table 1). As shown by the data presented in Table 2, compound 1 is an inhibitor of sEH enzymatic activity, inhibits calcium influx into Jurkat cells, inhibits IL-2 reporter gene function, blocks the production of IL-2, IL-4 and IFN γ by human PBMCs, inhibits IL-2 production in SEB challenged mice and exhibits immunosuppressive activity in the mouse allogeneic cell transplant model. This constellation of *in vitro* and *in vivo* biological activities exhibited by compound 1 demonstrates that inhibition of sEH enzymatic activity results in a profile consistent with immunosuppression.

To further strengthen the correlation between inhibition of sEH enzymatic activity and inhibition of T-lymphocyte activation, seven compounds belonging to the same chemical series as compound 1 and exhibiting a range of potencies, were tested for 1) inhibition of sEH enzymatic activity, 2) inhibition of calcium influx into Jurkat cells and 3) inhibition of IL-2 reporter gene function. As shown by the experimental results presented by Table 3, the more potent compounds achieved the greatest efficacy in the cellular assays. This provides strong evidence that inhibition of sEH activity will prevent activation of Jurkat cells. A known inhibitor of sEH, 4-phenyl chalcone oxide, was also shown to weakly inhibit calcium influx into Jurkat cells, providing additional evidence which links sEH functional activity with T-lymphocyte function.

Direct evidence that inhibition of sEH will also inhibit the activation of primary human T-lymphocytes (PBMCs) was obtained by comparing inhibition of sEH enzymatic activity at 1 μ M with inhibition of IL-2 production by human whole blood at 2 μ M. Three additional compounds derived from this chemical series were tested in this fashion and, as shown by Figure 1, inhibition of sEH by these compounds did indeed tightly correlate with inhibition of IL-2 release from human whole blood cells. One could therefore predict from these data that administration of an inhibitor of sEH, in a sufficient

amount and therapeutically suitable dosage form, would result in immunosuppression and therefore be of clinical benefit for treating immunological disorders.

Experimental Methods

Soluble Epoxide Hydrolase Enzyme Assay (s-NEPC colorimetric assay):

Soluble epoxide hydrolase is responsible for the hydrolysis of epoxides to their subsequent diols. Classically the determination of this activity employs radiolabeled *trans*-stilbene oxide as the standard substrate. More recently, a new class of spectrophotometric substrates for sEH have been synthesized, one being s-NEPC (4-nitrophenyl (2S,3S)-2,3-epoxy-3-phenylpropyl carbonate) (Dietze et al., Analytical Biochemistry, 216:176-187;1994). s-NEPC contains both an epoxide and a 4-nitrophenol moiety. Enzymatic hydrolysis of the s-NEPC quantitatively releases the 4-nitrophenol which was subsequently monitored at 405 nm.

Soluble epoxide hydrolase (partially purified from human liver) initiated the enzymatic reaction after addition to substrate and compounds, 25 μ M s-NEPC in the presence or absence of compounds in 0.1 M sodium potassium phosphate buffer at pH 6.4. The final volume of the assay was 1 ml. A stock solution of s-NEPC substrate was prepared fresh daily in absolute ethanol and held on ice during use. Initial rates of hydrolysis were measured to determine inhibition of enzyme activity.

Cytosolic Soluble Epoxide Hydrolase Enzyme Assay (EET assay; Method I):

The cytochromes P450 are capable of forming various arachidonic acid metabolites, including epoxyeicosatrienoic acids (EETs). The EETs are the best known endogenous substrates of sEH. Soluble epoxide hydrolase activity in 145,000 x g cytosolic supernatants was employed to enzymatically convert (+/-) 14,15-EET to its corresponding diol, (+/-) 14, 15-dihydroxyeicosatrienoic acid (DiHET), in order to examine the inhibitory potential of test compounds against sEH. Incubations (1ml total volume) consisting of 10 μ M (+/-) 14, 15-EET and either 100 μ g of cytosolic protein

(human liver) or 625 µg of cytosolic protein (Jurkat cells) in 66 mM TRIS buffer pH 7.4 (*tris*-Hydroxymethyl-aminomethane) were run at 37°C for 3 minutes. The incubations were stopped with 25 µl of glacial acetic acid and the (+/-) 14, 15-EET and (+/-) 14, 15-DiHET were extracted into diethyl ether. The extract was dried under nitrogen and the residue solubilized and then injected onto an RP-HPLC for quantitation of (+/-) 14,15-EET and (+/-) 14, 15-DiHET (Symmetry 4.6 x 100mm C18 column (Waters Corp., Milford, MA) developed with a 70:30 A:B to 85:15 A:B gradient over 15 minutes at 40°C; A = methanol + 0.028% TFA (trifluoroacetic acid) and B = water + 0.028% TFA). These control incubations were compared to similar incubations that contained test compound. The percent inhibition of sEH was reported as the difference in the amount of (+/-) 14, 15-DiHET formed during the incubations with or without the test compound inhibitor.

Soluble Epoxide Hydrolase Enzyme Assay (EET assay; Method II):

Recombinant human sEH (preparation described below) was employed to convert (+/-) 14,15-epoxyeicosatrienoic acid (EET) to its corresponding diol, (+/-) 14, 15-dihydroxyeicosatrienoic acid (DiHET), in order to examine the inhibitory potential of test compounds against sEH. The test compounds (1 µM) were pre-incubated with sEH (40 nM) in 250 µl of 20mM TES (2-[[*tris*(Hydroxymethyl)methyl]amino]ethanesulfonic acid) buffer containing 200mM NaCl, pH 7.5 for one hour. The enzymatic reaction was initiated with the addition of (+/-) 14,15 EET (10 µM) and incubated for five minutes at room temperature. Ten seconds prior to the completion of the reaction, an internal standard, 9,10 EODE (1.3 µM), was added. The reaction was quenched by addition of 500 µl ethyl acetate. The test tubes were centrifuged for five minutes at 2500 rpm and the top layer was removed and transferred to an amber septa vial with a polyspring insert. The tubes were then placed under vacuum until dry. The samples were re-suspended in 30 µl absolute ethanol and then analyzed by LC-MS. The peak areas of the (+/-) 14, 15 EET and (+/-) 14, 15 DiHET detected by the LC-MS were integrated using the LC-MS software (Micromass Masslynx version 3.3; Manchester, United Kingdom). Conversion of substrate was calculated as the ratio of (+/-) 14,15 DiHET to (+/-) 14,15 EET. The

percent inhibition of sEH was reported as the ratio of the conversion of substrate with test compounds to the conversion measured in the control.

LC/MS Analysis of 14, 15 EET Conversion to 14, 15, DiHETE by sEH:

A gradient, reverse phase, HPLC method was employed to separate (+/-) 14,15, EET from its enzymatic conversion product (+/-) 14, 15-DiHETE. The method employed ammonium acetate buffer (neutral pH) to avoid conversion due to acid catalysis. Mass spectrometry with electrospray ionization was employed for detection of the two analytes and an internal standard (9, 10 EODE). The internal standard serves to monitor sample recovery during extraction, and work-up, as well as, to compensate for fluctuations in instrument response. The ions at $m/z = 314.25$ (IS), 338.3 (EET), and 356.3 (DiHETE) were monitored under selected ion monitoring instrument control; these ions are formed from the respective analyte and internal standard molecules by adduction of NH_4^+ formed from the HPLC buffer. Samples are supplied, in ethanol solution, in amber vials with low dead volume inserts compatible with the HP 1050 HPLC autosampler.

HPLC Methodology

Instrument: HP 1050, with autosampler (Hewlett Packard, Wilmington, DE). Column: LUNA 5 μ C8 30 x 2.0 mm # 00A4249-BO (Phenomenex, Torrance, CA). A mobile phase 50 μ l acetonitrile, 950 ml water, 750 mg ammonium acetate, B mobile phase 900 ml acetonitrile, 100 ml water 750 mg ammonium acetate. Initial conditions: 85%A 15%B at 300 μ l/min. programmed to 99% B, 1% A at 5 minutes, hold to 7 minutes, return to initial conditions at 9 minutes, equilibrate to 15 minutes. Injection volume was 5 μ l.

MS Methodology

Instrument: Micromass Platform LCZ (Micromass, Manchester, United Kingdom). Electrospray ionization was employed with a column effluent split of 10:1. Ions at 314.3, 338.25, 356.25 monitored with a 0.5 sec dwell, 0.02s inter ion time. Cone voltage 20V, resolution 14.5 HM, 14.5 LM (approximate unit mass). Multiplier 551, source temp 100°C, Desolvation 200°C.

Cloning, Expression and Purification of Recombinant Human sEH:

A human sEH gene sequence was PCR amplified from a human spleen cDNA library (Marathon-Ready, RACE-PCR, Clontech, Palo Alto, CA). The resultant PCR product was T-A tail ligated into pGem-T (Promega, Madison, WI) and subsequently transformed into competent JM109 cells. A clone containing an amino acid sequence identical to a published sEH sequence was identified through DNA sequencing (Sandberg and Meijer, *Biochem. Biophys. Res. Commun.* **221**:333-339; 1996). This clone was then used to subclone sEH (in-frame) into a GST-fusion baculovirus expression vector, pAcG2T (PharMingen, San Diego, CA). The pGemT-sEH clone was digested with NdeI, the ends filled-in using DNA Polymerase I (Klenow large fragment; New England BioLabs, Beverly, MA) and the sEH fragment purified. The sEH fragment was ligated to pAcG2T digested with SmaI and transformed into competent JM109 cells. Colonies were screened and a clone containing the correct sequence was used for transfection and expression in baculovirus. Western Blot analysis was performed to confirm the expression of GST-sEH.

A suspension culture of SF9 insect cells grown in SF900 II SFM media containing 1% FBS, and 1% antibiotic/antimycotic (Gibco, Grand Island, NY) was inoculated into a stirred tank fermenter and grown to a cell density of approximately 1×10^6 cells/ml. The cells were then infected with the virus stock at a Multiplicity of Infection (MOI) equal to one. Seventy-two hours after infection the cells were harvested by centrifugation and stored at -80°C .

Insect cells containing the expressed GST-sEH fusion protein were resuspended in cold Run Buffer (50mM Tris pH 8.2, 1mM DTT, 0.1 mM EDTA, 10% glycerol, 150mM NaCl) containing 2mM MgCl_2 , 100 U/ml Benzonase (EM Sciences, Gibbstown, NJ) and protease inhibitors, and lysed using a Nitrogen bomb. Clarified supernates were applied to a Glutathione agarose column (Sigma, St. Louis MO), the column washed with Run Buffer until baseline absorbance achieved, and then the column was warmed to room temperature. Two column volumes of Cleavage Buffer (Run Buffer containing 2.5 mM CaCl_2 , 20 U/ml thrombin (USB, Cleveland OH) were applied to the column and allowed

to remain for two hours. The thrombin-cleaved sEH was eluted with Run Buffer, chilled, analyzed by SDS-PAGE, and stored at -80°C. Protein concentration was determined using a Coomassie Dye Binding Assay (Bio-Rad, Hercules, CA).

Calcium Influx Assay:

Jurkat cells were pelleted by centrifugation and washed twice with RPMI 1640 growth media containing 10 mM HEPES buffer and 10 % fetal bovine serum. The cells were then resuspended at 2×10^7 cells/ml in RPMI 1640 growth media containing 10 mM HEPES and 10 % fetal bovine serum. An equal volume of Fluo-3-AM (Molecular Probes Inc., Eugene, OR) and Pluronic F127 (Molecular Probes) was added to the cell suspension, to a final concentration of 2 μ M Fluo-3-AM and 0.2% Pluronic F127, and incubated for 45 minutes in the dark at room temperature. Between 20-30 ml of RPMI 1640 growth media containing 10 mM HEPES buffer and 10 % fetal bovine serum was added and the suspension incubated as before for an additional 15 minutes. The cells were then pelleted, washed cells 3 times with Hanks' Balanced Salt Solution (HBSS) containing 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 2 mM probenecid (Sigma, St. Louis MO), 1 % fetal bovine serum at pH 7.4, and resuspended at 2×10^7 cells/ml in the same buffer. The assay was conducted in 96-well black viewplates (Packard Instrument Company, Meriden, CT) where Jurkat cells (final concentration 3×10^5 / ml) were pre incubated with test compound for 15 minutes at room temperature, followed by addition of Anti-CD3 (clone X35, cat. # 0178, Immunotech; Beckman Coulter, Inc., Fullerton, CA) at 0.25 μ g/ml and fluorescence intensity was continuously monitored for 13 min measured using a Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 488 nm and emission wavelength of 535 nm. Calcium release was calculated from the difference in fluorescence intensity measured between baseline values and peak height. These values are plotted versus compound concentration to obtain an EC_{50} value for the compound's ability to inhibit calcium release. A decrease in Fluo-3-AM fluorescence intensity indicates inhibition of the release of calcium by the compound being tested (Minta et al., J. Biological Chemistry, 264:8171-8178;1989).

IL-2 Reporter Assay:

The IL-2 reporter assay measures transcriptional activation of a luciferase reporter gene that was placed under control of the IL-2 promoter/enhancer. All the known regulatory features of the IL-2 gene are contained within a ~300 bp sequence immediately upstream of the open reading frame. The region -328 to +35 relative to the transcription start site of the IL-2 gene was obtained by RT-PCR of human genomic DNA and was subcloned into the promoterless luciferase reporter vector pGL2-Basic (Promega, Madison, WI). The resulting construct, pIL2P-luc, and a vector containing a neomycin resistance gene, pcDNA/Neo (Invitrogen, Carlsbad, CA), were linearized and stably transfected into Jurkat cells (a human T-lymphocyte line) by electroporation. Following G-418 selection and dilution cloning, a Jurkat cell line was established, J.1F/C6, which exhibited a strong induction of luciferase activity upon treatment with ionomycin and PMA (up to 100-fold). This induction of luciferase activity in J.1F/C6 cells was inhibited by FK506, a drug which is a potent inhibitor of IL-2 expression by T-lymphocytes and currently used for preventing allograft rejection.

An assay was established employing J.1F/C6 cells for determining the activity of compounds for inhibiting IL-2 promoter driven luciferase activity. J.1F/C6 cells were pelleted by centrifugation, washed once with PBS, resuspended in RPMI (phenol red-free) containing 5% FBS, and dispensed into 96-well, white microtiter plates (Packard Instrument Company, Meriden, CT) at 50,000 cells/well. The cells were pre-incubated with test compounds (0.001, 0.01, 0.1, 1.0 and 10 µg/ml) for 15 min prior to addition of ionomycin (1 µg/ml) and PMA (10 ng/ml) in a final volume of 100 µl. Following a 6 hr incubation at 37°C in a humidified incubator, 100 µl of Luc-Lite lysis buffer/luciferase assay buffer (Promega, Madison, WI) was added and luminescence measured using a TopCount scintillation counter/luminometer (Packard Instrument Company, Meriden, CT). A drug-induced reduction in the functional activity of the IL-2 promoter results in inhibition of luciferase activity and consequently a decrease in luminescence.

IL-2 Production Assay (80% human whole blood):

Human peripheral blood was obtained from healthy donors by venipuncture (heparin used as anti-coagulant) and dispensed into test tubes. Test compound was added directly to the undiluted blood at 2.5 μ M, and aliquots dispensed into 96 well microtiter plates. Following a 20 minute preincubation at 37°C, staphylococcal enterotoxin B (cat # S-4881; Sigma, St. Louis, MO) in RPM I 1640 growth media containing 10 mM HEPES was added to each well. The final assay volume was 200 μ l per well with a final concentration of 2 μ M for each test compound. The plates were incubated for 23 hours at 37°C in a humidified incubator, plates were centrifuged to recover the supernatant plasma, and plasma assayed for IL-2 content using a commercial Human IL-2 ELISA kit (R&D Systems, Minneapolis, MN). The IL-2 content of each set of compound dosed wells was expressed as percent inhibition compared to IL-2 levels in vehicle control wells (0.5% dimethyl sulfoxide).

IL-2, IL-4 and IFN γ Production Assays (peripheral blood mononuclear cell):

Human peripheral blood was obtained from healthy donors by venipuncture and the peripheral blood mononuclear cell fraction (PBMCs) was prepared by centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradients. The cells were resuspended and dispensed in 96 well microtiter plates. Test compounds were added to the cells approximately 15 minutes prior to stimulation with ionomycin (1 μ g/ml) and PMA (10 ng/ml). The final volume of the assay was 100 μ l. Following a 16 hr incubation at 37°C, the cells were pelleted by centrifugation, and the supernatants were collected and stored at -70°C until the IL-2, IL-4 and IFN γ levels were quantitated by using commercial ELISA kits (Genzyme Diagnostics, Cambridge, MA).

Inhibition of Staphylococcal Enterotoxin B (SEB) Response in Mice:

Bacterial superantigens, such as staphylococcal enterotoxin B (SEB), polyclonally activate T-lymphocyte populations by cross-linking major histocompatibility complex (MHC) class II molecules on antigen presenting cells with the V β chain of the T-lymphocyte receptor. This mode of stimulation is through non-polymorphic regions of

class II and T-lymphocyte receptor molecules, and thus does not lead to "adaptive" immunity but rather induces massive production of cytokines (*e.g.* IL-2) by a large population of Class II-restricted, CD4-bearing T-lymphocytes. Superantigen in murine animal models has been used both *in vitro* and *in vivo* for analysis of endogenous IL-2 production from T-lymphocytes, and it has been an important method for establishing "proof of principle" efficacy for compounds designed to inhibit T-lymphocyte proliferation and activation.

Experiments were conducted *in vivo* using female BALB/c mice from 6-8 weeks of age (18-25 grams), although most mouse strains are suitable for studies. A minimum of one week for stabilization and conditioning was usually required before using the mice. Each study utilized approximately 32-48 recipient mice divided into groups of eight. Previous studies suggest that this is the minimum number of animals, which yields statistically significant results.

Polyclonal T-lymphocyte activation was readily induced *in vivo* in BALB/c mice by the intraperitoneal (i.p.) injection of SEB (5 µg/mouse in saline). The increase in plasma IL-2 levels, indicative of T-lymphocyte activation, became maximal three hours following SEB administration, at which time blood from individual mice was collected in ethylenediamine-tetraacetic acid (EDTA)-containing tubes to prevent clotting. The EDTA-treated blood samples were centrifuged to pellet leukocytes and red blood cells, and the plasma supernatant samples from individual mice (diluted 1:10 in PBS) were assayed by ELISA kit (Genzyme Diagnostics, Cambridge, MA) to quantitate IL-2 concentrations. Groups of mice receiving putative immunosuppressive agents were dosed subcutaneously, intraperitoneally or orally one hour prior to SEB administration. The SEB-induced IL-2 response was inhibited 80-90% by oral administration of 50 mg/kg cyclosporin A (CsA), an inhibitor of T-lymphocyte activation. The Student's t-test was used to determine significant differences between groups of untreated mice and those mice treated with putative immunosuppressive agents.

Allogeneic Cell Transplant Response in Mice:

The ability of the immune system to distinguish between cells from self and cells from genetically different individuals (non-self) is necessary to maintain physiological homeostasis. The allogeneic cell transplant response is therefore an important model for studies of transplant rejection. This T-lymphocyte mediated immune response can be induced in adult mice by the injection of lymphocytes from a non-histocompatible mouse strain. This response is characterized by T-lymphocyte proliferation, which is limited to the popliteal lymph node that receives drainage from the footpad area. No *in vitro* system exists that can exactly duplicate this *in vivo* response. The assay is commonly used to evaluate new immunosuppressive molecules. This assay is preferred because the magnitude of the response is significantly greater than the local GVH response in mice (Krocze et al., J. Immunology, 139:3597-3603;1987).

Experiments were conducted using male or female mice (20-26 grams). Any non-histocompatible mouse strains suffice for donor and recipient populations. Typically DBA mice were used as donors and C57Bl/6 mice were used as recipients. A minimum of one week stabilization and conditioning period was usually required before use of the mice. Each study utilized approximately 36 recipient mice divided into groups of six. Previous studies suggested that this is the minimum number of animals, which yields statistically significant results.

Donor mice were sacrificed by CO₂ asphyxiation and spleens were removed and a cell suspension was prepared. The cell suspension (1.0×10^7 /metatarsal in 0.05 ml) was injected into the dorsal metatarsal skin of recipient mice. Four days later, the animals were sacrificed by CO₂ asphyxiation and the popliteal nodes were removed and weighed. Groups of mice receiving putative immunosuppressive agents were dosed subcutaneously, intraperitoneally or orally one hour prior to cell injection and daily thereafter. The tests lasted approximately four days. The assay involved no footpad swelling and only a moderate increase in the size of the popliteal lymph node. The Student's t-test was used to determine significant differences of popliteal lymph nodes'

weights between groups of untreated mice and those mice treated with putative immunosuppressive agents.

WHAT IS CLAIMED IS:

1. A method for treating a patient suffering from an immunological disorder, comprising administering to the patient a therapeutically effective amount of a soluble epoxide hydrolase inhibitor, either alone or in combination with other therapeutic agents.
2. A method for treating a patient suffering from an autoimmune disease, comprising administering to the patient a therapeutically effective amount of a soluble epoxide hydrolase inhibitor, either alone or in combination with other therapeutic agents.
3. A method for treating a patient suffering from a disorder associated with T-lymphocyte mediated immune responses comprising administering to the patient a therapeutically effective amount of a soluble epoxide hydrolase inhibitor, either alone or in combination with other therapeutic agents.
4. A method for treating transplantation, allograft xenograft rejection comprising administering to the patient a therapeutically effective amount of a soluble epoxide hydrolase inhibitor, either alone or in combination with other therapeutic agents.
5. A method for preventing graft versus host disease comprising administering to the patient a therapeutically effective amount of a soluble epoxide hydrolase inhibitor, either alone or in combination with other therapeutic agents.
6. The method as recited in claim 1 wherein the soluble epoxide hydrolase inhibitors is selected from the group consisting of *N*-[4-(5-ethyl-3-pyridin-3-yl-pyrazol-1-yl)-phenyl]-nicotinamide; *N*-[4-(5-Ethyl-3-pyridin-3-yl-pyrazol-1-yl)phenyl]-1-methylindole-2-carboxamide; 4-(3-Cyanopropoxy)-*N*-[4-(5-cyano-3-pyridin-3-yl-pyrazol-1-yl)phenyl]benzamide; and *N*-[4-(5-cyano-3-pyridin-3-yl-pyrazol-1-yl)phenyl]-4-(3-[1,3]dioxolan-2-yl-propoxy)benzamide
7. The method as recited in claim 2.

8. The method as recited in claim 3.
9. The method as recited in claim 4.
10. The method as recited in claim 5.

1/1

Figure 1: Correlation between Inhibition of IL-2 Production and sEH